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Biogenesis of the Gram-Negative Bacterial Envelope

Review

Franck Duong, Jerry Eichler, Albert Price, Marilyn Rice Leonard, and William Wickner
Department of Biochemistry
Dartmouth Medical School
Hanover, New Hampshire 03755-3844

The cell envelope of gram-negative bacteria is a 3-layered structure consisting of a bilayer-based plasma membrane, a periplasm of soluble proteins, peptidoglycan mesh, and membrane-derived oligosaccharide, and an outer membrane with proteins and lipids of unique structure (Figure 1A). It offers opportunities for the study of protein and lipid topogenesis, as well as a fertile, proven area for antibacterial drug discovery. For many of the events that underlie cell envelope growth, mechanistic studies are still in their infancy.

The bacterial plasma (inner) membrane has the selective nutrient transport functions of the eukaryotic plasma membrane, the protein translocation and lipid biosynthetic functions of the endoplasmic reticulum, and the oxidative phosphorylation function of the mitochondrial inner membrane. Its lipid bilayer is comprised of phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol, synthesized by enzymes of the Kennedy pathway (Raetz and Dowhan, 1990). In contrast to outer membrane proteins, the membrane-spanning segments of inner membrane integral proteins are largely α-helical and apolar, whether for a single span that can anchor a protein with a large polar domain or for the multiple membrane-spanning helices of transport proteins.

The periplasm is an aqueous space between the inner and outer membrane. It contains membrane-derived oligosaccharide (Kennedy, 1987), soluble "binding protein" components of transport systems, chaperones, and oligo-nucleotide, -peptide, and -saccharide hydrolases that fulfill a lysosomal function. In addition, its peptidoglycan meshwork provides cell shape and anchors the outer membrane lipoprotein (Braun and Sieglin, 1970).

The outer membrane has a structure and composition that is unique to bacteria in several regards. Its most abundant proteins are peptidoglycan-linked lipoproteins with characteristic covalent lipid modifications and porins, which are oligomeric β-barrel structures with large aqueous channels. The lipid bilayer of the outer membrane is asymmetric, with an inner leaflet of the same three glycerophospholipids as the inner membrane and an outer leaflet of lipopolysaccharide (Raetz, 1993). Pili and flagella, complex filamentous structures, protrude from the cell surface.

This review of our current knowledge of how newly made cell surface components are sorted to their correct compartment will focus on translocation across the inner membrane and only briefly consider the biogenesis of the outer layers. These studies have been immeasurably enriched by the excellent genetics, facile separation of compartments, growth in defined minimal media, and advanced enzymology of E. coli.

Inner Membrane Translocation

Large polar proteins or polar domains of integral membrane proteins cross the membrane through preprotein translocase (Figure 1B). Proteins that use translocase include permanent residents of the periplasm, proteins en route to the outer membrane or cell exterior, and large polar periplasmic domains of integral inner membrane proteins. Most are synthesized in precursor form ("preproteins") with an amino-terminal leader (signal, pre-) sequence (Randall and Hardy, 1989) preceeding their "mature" domain. Leader peptide regions are typically 16–23 residues long, with an aminoterminal basic domain, a central apolar domain of 8–12 residues, and a C-terminal domain with the leader peptidase recognition site. Genetic and biochemical studies have shown the importance of each of these features. Though each leader region functions optimally with its natural mature protein partner, leader regions retain at least some function when transferred to another mature domain (Tommassen et al., 1983). They have been shown to: (1) inhibit the folding of the mature domain (Liu et al., 1989), thereby allowing capture by chaperones such as SecB and preventing the cytoplasmic formation of stable structures that could "jam" the translocase (Collier et al., 1988); (2) bind to the SecA subunit of translocase (Lill et al., 1990); and (3) specify proteolytic cleavage by leader peptidase or lipoprotein specific signal peptidase at the periplasmic surface of the inner membrane. Preprotein leader domains could also partition spontaneously into lipid bilayers (Briggs et al., 1985; Batenburg et al., 1988), though it remains unclear whether this occurs in the normal sequence of events that leads to translocation.

Targeting to the Inner Membrane

Presecretory proteins are sorted from their cytosolic counterparts and delivered to the plasma membrane by a complex cascade of chaperones. Two ribosome-bound chaperones, the "trigger factor" prolyl isomerase (Guthrie and Wickner, 1990; Stoller et al., 1995) and SRP, the "signal recognition particle" (Poritz et al., 1990; Luirink et al., 1994; Miller et al., 1994), compete for nascent proteins as they first emerge from the ribosome (Valent et al., 1995). Biochemical studies suggest that proteins without signal sequences are captured by trigger factor, which may deliver them to the cavity of GroEL for folding (Kandror et al., 1995). Some membrane proteins may escape GroEL folding by the association of SRP with their leader sequence (Luirink et al., 1994) and then be released by FtsY interaction with the Ffh subunit of the SRP (Miller et al., 1994). This mechanism may have only a minor effect on proteins that are ultimately exported to the periplasm or outer membrane, as the absence of SRP has no measurable effect on their export (Ulbrandt et al., 1997). In contrast, the integration of certain proteins into the plasma membrane depends on SRP (ibid.). Further studies are needed to determine whether SRP slows the synthesis of these proteins, modulates their folding, or participates in their delivery to other chaperones or to translocase itself. Certain preproteins are captured by SecB, a tetrameric, export-dedicated chaperone (Collier et al., 1988; Gannon et al.,

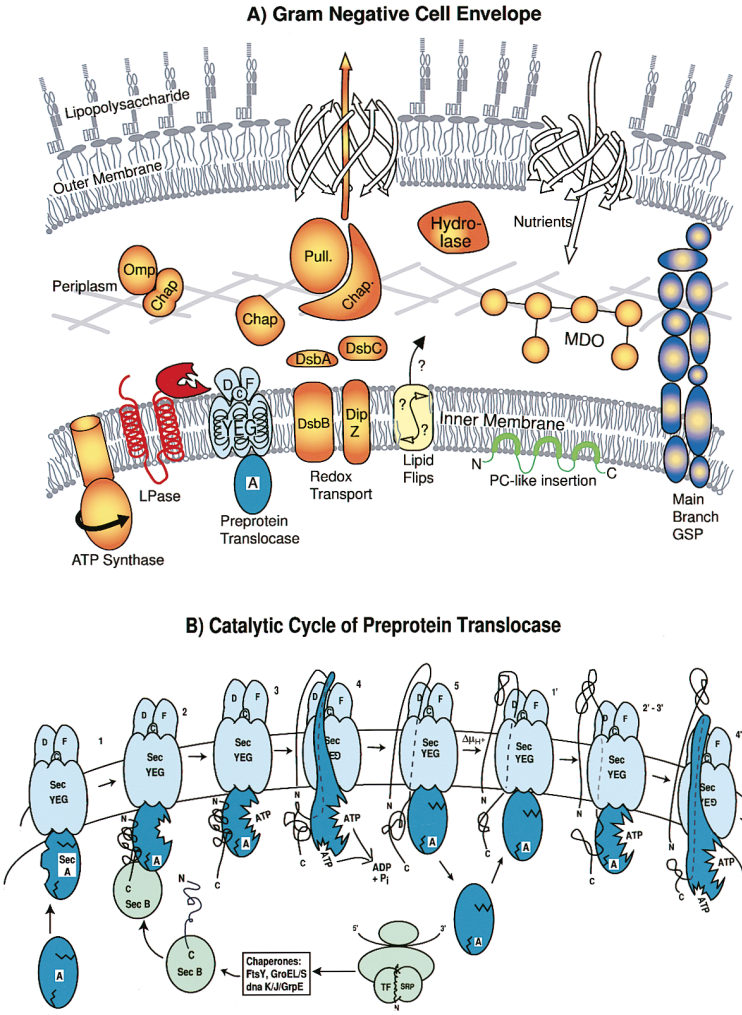

Figure 1. The Cell Surface of E. coli

(A) The envelope of E. coli, with particular emphasis on elements of topogenesis discussed in the text. Abbreviations: Omp, outer membrane protein; Pull, pullulanase; Chap, chaperone; MDO, membrane-derived oligosaccharide; LPase, leader peptidase; Dsb, genes that affect the formation of correct disulfide bonds; PC, M13 procoat; GSP, general secretory pathway. Though not depicted, there is a proton motive force across the inner membrane, positive outside, which drives ATP synthesis, the transport of many solutes, and (step 5 to 1′ in [B]) forward movement of preprotein translocation intermediates.

(B) Catalytic cycle of preprotein translocase, a SecA dimer bound at the membrane-embedded heterohexameric SecYEGDFyajC. See the text for details. The smallest subunit is the product of the *yajC* gene and is referred to as yajC rather than YajC to avoid confusion with SecY.

1989; Hardy and Randall, 1991). SecB associates with the mature domain of preproteins through a "kinetic partitioning" recognition of proteins that are slow to fold (Randall et al., 1994), prevents their folding into an export-incompatible structure and, through its affinity for the SecA subunit of translocase (Hartl et al., 1990), participates in their productive membrane targeting.

Preprotein Translocase

Preprotein translocase is a complex, multisubunit protein consisting of a dimer of SecA, the peripheral domain of translocase, bound stoichiometrically to a multisubunit, membrane-embedded domain (Figure 1B). This integral domain of translocase is formed from SecY, SecE, SecG, SecD, SecF, and yajC subunits (Duong and Wickner, 1997a). SecY and SecE are conserved throughout evolution, having their physical and functional homologs in yeast and mammalian endoplasmic reticulum and even in primitive mitochondria (Stirling et al., 1992; Panzner et al., 1995; Lang et al., 1997). These two subunits are indispensable for cell growth and together form a minimal functional core of translocase (Duong and Wickner, 1997a). The SecYE core is fully capable of binding SecA and activating its ATPase activity (see below), yet without an additional integral membrane partner, it is very inefficient at supporting translocation. SecYE is joined, both physically and functionally, either with SecG, with a trimeric complex of SecD, SecF, and yajC (encoded together in one operon), or with both SecG and SecDFyajC. These partner subunits for SecYE are apparently not found in eukaryotic translocases. Understanding their functions has required an understanding of the other uniquely prokaryotic translocase subunit, SecA.

SecA is a dimer (Akita et al., 1991; Cabelli et al., 1991) of 102 kDa subunits, polar in sequence and water-soluble at even high concentrations. SecA is found both in the cytoplasm and bound to the membrane (Oliver and Beckwith, 1982), and indeed functions both as a repressor of its own translation (Dolan and Oliver, 1991) and as an essential subunit of translocase. Its high-affinity membrane binding (Hartl et al., 1990) requires both its affinity for acidic lipids and for the SecYE core of the integral translocase domain (Hendrick and Wickner, 1991). It also binds to nonsaturable lipid sites (Lill et al., 1990; Breukink et al., 1992; Ulbrandt et al., 1992; Chen et al., 1996; Eichler and Wickner, 1997), though (as noted below) this binding is not associated with preprotein translocation. SecA has two nucleotide-binding domains, NBD1 and NBD2 (Mitchell and Oliver, 1993). NBD1 alone can support the SecA cycle of insertion and

deinsertion (Economou et al., 1995), as described below, but NBD2 has an essential role in coupling preprotein movement to this SecA cycle (Mitchell and Oliver, 1993; Economou et al., 1995). SecA has been shown to interact directly with ATP (Lill et al., 1989), SecB (Hartl et al., 1990), the leader regions and mature domains of preproteins (Lill et al., 1990), acidic lipids, and SecYEGDFyajC (Duong and Wickner, 1997a). Each of these interactions is apparently exquisitely regulated to guide the successive stages of the translocase catalytic cycle. Upon binding to acidic lipid and to the SecYE core of SecYEGDFyajC (Figure 1B, step 1), SecA is activated for its recognition of (1) SecB and (2) the leader and (3) mature domains of preproteins (Hartl et al., 1990). Thus, preprotein/SecB complexes are guided to translocase (Figure 1B, step 2) by these 3 separable recognition events, though recognition of either the leader alone (Summers et al., 1989) or the mature domain alone (Derman et al., 1993) can sometimes suffice. Preprotein association in turn activates SecA for the binding and hydrolysis of ATP (Lill et al., 1989). Upon binding ATP at the high-affinity NBD1 site (step 3), SecA undergoes a profound conformational alteration, inserting large domains of 65 and 30 kDa, near the N and C termini, respectively, into the membrane (step 4). SecA inserts through the SecYEGDFyajC integral membrane complex, largely shielded from the fatty acyl phase of the membrane (Economou and Wickner, 1994; Price et al., 1996; Eichler and Wickner, 1997; Eichler et al., 1997). SecA insertion carries the first N-terminal loop of preprotein across the membrane. It is not known whether the leader region per se is exposed to the lipid at this point. Even a single SecA insertion event can support sufficient preprotein translocation to allow leader peptidase cleavage at the periplasmic surface of the membrane (Schiebel et al., 1991). SecA then hydrolyzes ATP, releases the partially translocated preprotein, and (with additional ATP binding and hydrolysis) deinserts (step 5; Economou and Wickner, 1994). The role of ATP hydrolysis is established by experiments in which SecA cannot hydrolyze its bound ATP, either because of a mutation in the NBD1 region of the *secA* gene that prevents SecA ATP hydrolysis, or because a nonhydrolyzable ATP analog is used in place of ATP. In either case, SecA is locked in the inserted state and remains bound to preprotein (Schiebel et al., 1991; Economou et al., 1995). However, under normal conditions and in that phase of the reaction cycle where SecA has deinserted, the preprotein chain is free to slide through the integral domain of translocase, driven forward by $\Delta\mu_H+$ (step 5 to 1′) (Schiebel et al., 1991; Duong and Wickner, 1997b). The deinserted SecA is free to exchange with cytosolic SecA (Economou et al., 1995). SecA can also re-engage the next untranslocated portion of the preprotein and, upon binding a fresh ATP, drive another "loopful" of the preprotein across the membrane (steps 1′ to 4′). Indeed, for I_{16} and I_{26}, the abundant kinetic intermediates of translocation of proOmpA (the precursor form of outer membrane protein A) in which 16 or 26 kDa of the preprotein has translocated, the addition of a nonhydrolyzable ATP analog is sufficient to drive both a limited translocation of a few kilodaltons of preprotein as well as SecA insertion (Schiebel et al., 1991). Conditions of varied ATP concentrations, $\Delta\mu_H+$, SecYEGDFyajC subunit composition, or denaturant extraction that modulate SecA insertion or deinsertion cause a corresponding forward or reverse movement of the preprotein (Duong and Wickner, 1997b), establishing that the two movements are closely coupled. SecA cycling occurs throughout the movement of the preprotein, not merely at the initiation or termination of translocation (Duong and Wickner, 1997b; Eichler and Wickner, 1997). Regions of SecA can also undergo conformational change and even become protease resistant upon interaction with lipid and ATP (Shinkai et al., 1991; Ulbrandt et al., 1992; Eichler and Wickner, 1997), but this conformational change differs from that seen during translocation in several important respects (Eichler and Wickner, 1997): (1) it does not need preprotein; (2) it does not need SecYEGDFyajC, which is essential for preprotein translocation; (3) protease-resistant, lipid-associated 35S-SecA does not "chase" upon addition of excess nonradioactive SecA during translocation (Chen et al., 1996); (4) protease resistance is not lost upon disruption of the membrane permeability barrier. This SecA/lipid association and conformational change has not been shown to be related to translocation, and it remains unclear how much it occurs in vivo.

The conserved SecY and SecE subunits alone can serve as a high-affinity receptor for SecA, can activate SecA for preprotein binding and ATP hydrolysis, and can even function as a (inefficient) two-subunit integral domain of translocase (Kawasaki et al., 1993; Duong and Wickner, 1997a), though their roles have been questioned (Yang et al., 1997). Since SecG and SecDFyajC are specific for bacteria, one may wonder what function they fulfill. It appears that they are specifically dedicated to promoting the cycle of SecA insertion and deinsertion. SecG inverts its membrane topology (the inverted G in step 4 of Figure 1B) as SecA inserts and "flips" back as SecA deinserts (Nishiyama et al., 1996), promoting the insertion and deinsertion of SecA (Duong and Wickner, 1997a). SecDFyajC stabilizes the inserted form of SecA (Economou et al., 1995; Duong and Wickner, 1997a), and the major proOmpA translocation intermediates I_{16} and I_{26} only accumulate due to the SecDFyajC-mediated stabilization of inserted SecA (Duong and Wickner, 1997b). These intermediates are efficiently swept forward to complete translocation by the $\Delta\mu_H+$, and indeed the translocase "leaks" protons in the absence of SecDFyajC (Arkowitz and Wickner, 1994). We still have little idea of how the $\Delta\mu_H+$ functions to drive preprotein movement. However, it is clear that each of the bacteria-specific subunits function together and in a fashion that uses the uniquely bacterial combination of energy sources for translocation, ATP on the *cis*-side of the translocase and $\Delta\mu_H+$ functioning late in the process.

The mechanism of SecA cycling is so unexpected, even bizarre, that independent means are needed to establish this mechanism. SecA has been shown to be accessible to membrane-impermeable chemical probes in spheroplasts (Kim et al., 1994; van der Does et al., 1996; Ramamurthy and Oliver, 1997). Studies with photoactivable crosslinkers bound at unique positions on preproteins arrested during translocation have shown that SecA remains adjacent to the preprotein throughout its membrane transit (Joly and Wickner, 1993). Furthermore, analogous mechanisms are found for other transport reactions. The first example, which preceded the

SecA studies, was the histidine permease of S. typhimurium, in which the hisP protein was first shown through allele-specific suppression to interact with the periplasmic histidine-binding protein, then found to be modified by membrane-impermeant probes added to spheroplasts (Baichwal et al., 1993). More recently, the ATP-binding MalK subunit of the maltose transporter has been shown to be accessible from the periplasm (Schneider et al., 1995) and the KspT subunit of the polysialic acid transporter of E. coli has been shown to undergo cycles of membrane insertion and deinsertion (Bliss and Silver, 1997). In each case, the transport system has a peripherally bound subunit with an ATP binding domain. Further studies are needed to establish the generality of this mechanism in other transport systems.

Despite these advances, our understanding of preprotein translocase has not reached a chemical level. Determination of the structures of SecA (J. Hunt, J. Deisenhofer, and D. Oliver, personal communication), SecB, and SecYEGDFyajC will provide an essential foundation for future studies. Other proteins such as FtsH, which have been implicated in the translocation arrest of integral membrane proteins (Akiyama et al., 1994), might also be part of the SecYEGDFyajC complex. Also, little is known of the dynamic changes in translocase structure during its catalytic cycle (Figure 1B). For example, we do not know whether both subunits of the SecA dimer insert at the same time, whether transmembrane "loops" of subunits other than SecG also invert during catalysis, or whether subunits exchange during catalysis. Since (1) SecDFyajC is substoichiometric to SecYEG, (2) all proOmpA chains pass through the abundant translocation intermediates I_{16} and I_{26} (Schiebel et al., 1991), and (3) these translocation intermediates require SecDFyajC for stabilization (Duong and Wickner, 1997b), the SecDFyajC may exchange between the more abundant SecYEG complexes. Finally, central catalytic questions remain unanswered: How does $\Delta\mu_H+$ drive translocation (steps 5 to 1′ of Figure 1B), since even uncharged preproteins can respond to this energy source (Kato et al., 1992)? How do apolar domains escape laterally from translocase into the lipid phase? How does SecA recognize the mature domain of exported proteins (Lill et al., 1990; Prinz et al., 1996), and what subtle feature is being recognized? We believe that structure determinations will help to frame experiments that may address these functional mysteries in the future.

Inner Membrane Proteins

Inner membrane proteins with only a small periplasmic domain may insert into the inner membrane and translocate these domains without the aid of preprotein translocase (Andersson and von Heijne, 1993). M13 coat protein is made as a precursor, termed "procoat," of 73 amino acyl residues with a typical N-terminal leader region, an acidic periplasmic domain, a 20 amino acyl residue membrane spanning domain, and a short basic cytoplasmic tail. Its membrane assembly (reviewed in Wickner, 1988) is independent of SecA and SecY in vivo and was reconstituted with simply liposomes bearing entrapped protease or leader peptidase. Translocation of the central polar domain requires the membrane electrochemical potential, the apolar domains of both the leader and mature region, and the basic character of both the N- and C-terminal cytoplasmic "tails." The requirement for the membrane potential, and indeed even for the leader region as a whole, was shown to be exquisitely dependent on the particular charged residues of the periplasmic loop (Rohrer and Kuhn, 1990). Nevertheless, these studies did not uncover the chaperones for this protein or determine whether the rate of its spontaneous membrane insertion could be enhanced by other, non-Sec protein catalysts. Other proteins with small periplasmic domains, such as the N terminus of leader peptidase (Whitley et al., 1995), the melibiose permease (Bassilana and Gwizdek, 1996), or propilin (Majdalani and Ippen-Ihler, 1996) also assemble without preprotein translocase (Figure 1A, in green). Andersson and von Heijne (1993) have shown that the size of the polar periplasmic loop is a primary determinant of translocase dependence, and that transmembrane topology is governed by the transmembrane distribution of basic residues, the "inside-positive" rule (von Heijne, 1989). This rule is apparently valid for both translocase-catalyzed and translocase-independent preprotein assembly into the membrane. Perhaps basic residues orient apolar domains through electrostatic interactions with the negatively charged phospholipids as they bind to the lipid bilayer or to a pocket on the translocase SecA subunit, while the membrane potential inhibits the spontaneous translocation of basic residues.

It has recently been discovered that bacterial signal recognition particle (SRP) is required for assembly of a subset of inner membrane proteins (de Gier et al., 1996; Ulbrandt et al., 1997; Seluanov and Bibi, 1997). These include certain multispanning proteins, such as α-ketoglutarate permease and cytochrome oxidase d, but not others, such as mannitol or maltose permeases or SecE (Ulbrandt et al., 1997). de Gier et al. (1996) have found that SRP is required for leader peptidase membrane assembly. This is noteworthy in that the membrane assembly of this protein is not coupled to ongoing protein synthesis (Wolfe and Wickner, 1984). Thus, the chaperone function of bacterial SRP, like its relative in the chloroplast (Li et al., 1995), may not imply a coupling of translocation to translation, as for mammalian SRP and its receptor in the endoplasmic reticulum. It remains to be shown whether FtsY, the bacterial homolog of the SRP receptor, or bacterial SRP have direct affinity for the SecA subunit of translocase and thus, like SecB, can function as targeting chaperones.

Periplasmic and Outer Membrane Transit

After (or during) inner membrane translocation, the leader region is removed by leader peptidase or lipoprotein signal peptidase. Disulfide bond formation is catalyzed in the periplasm by DsbA and DsbC (Missiakas and Raina, 1997) and certain proteins, such as lipoprotein, pilin, and pulD (Hardie et al., 1996), are chaperoned through the periplasm in their transit to the outer membrane and beyond (see Pugsley, 1993, for an excellent, thorough review). Proteins of the periplasm are, as a rule, folded into stable, protease-resistant conformations, consistant with the digestive nature of this compartment. The periplasm is an intermediate compartment for proteins that will continue on to the outer

membrane, but in most cases it is unclear whether proteins are targeted to integrate into the outer membrane rather than "back" into the inner membrane by their affinity for lipopolysaccharide or by other means. Proteins that integrate into the outer membrane have extensive β structure rather than apolar α helices which, as has been noted (MacIntyre et al., 1988; Pugsley, 1993), would have arrested their inner membrane transit. Secretion of a protein across the outer membrane to the cell exterior can require a large complex of inner membrane and periplasmic proteins as seen for pullulanase (Pugsley, 1993). This complex might have energy transduction or chaperone functions. Secretion across the outer membrane can also require dedicated transport proteins in the outer membrane, such as PapC for pilin export (Norgren et al., 1987) or PulD for pullulanase export (Hardie et al., 1996), which may function as specialized pores. Other proteins can support their own integration into the outer membrane and then undergo proteolytic release of a domain at the cell exterior (Klauser et al., 1992). Many proteins must be fully folded in the periplasm (if not oligomerized or in their final conformation) to be integrated into, or translocated across, the outer membrane (Hirst and Holmgren, 1987; Peek and Taylor, 1992; Pugsley, 1992). For this reason, protein integration into the outer membrane is quite sensitive to mutations that might alter folding, in stark contrast to protein secretion to the periplasm, which is insensitive to such changes.

Some proteins, such as hemolysin α (HlyA), are secreted by a Sec- and leader sequence-independent pathway, employing an outer membrane (TolC) protein, a dedicated, inner membrane (HlyB) translocating ATPase, and a membrane fusion protein (HlyD). HlyA is exported directly from the cell without a periplasmic intermediate (Koronakis and Hughes, 1994). Cell-free reconstitution studies will be a key to further understanding all forms of transit across the outer membrane. This may entail the isolation of sealed, inverted outer membrane vesicles and the purification of the translocation-competent periplasmic forms of outer membrane proteins with their chaperones.

Lipid Assembly

Despite the wealth of information about protein sorting, processing, and folding, almost nothing is known about how glycerophospholipids "flip" from the cytoplasmic leaflet of the inner membrane, where they are assembled, to the outer leaflet of that membrane or to the inner leaflet of the outer membrane (Figure 1A). Studies in B. megaterium (Rothman and Kennedy, 1977; Langley and Kennedy, 1979) showed that lipid flipped postsynthetically and in an energy-independent reaction. Inverted membrane vesicles would seem ideal for in vitro assays of lipid translocation, as the newly made phosphatidylethanolamine moves from being accessable to membrane-impermeable reagents like trinitrobenzene sulfonic acid to being inaccessable in the periplasmic leaflet. Similarly, it is not known how lipopolysaccharide is delivered to the outer membrane. These problems appear ripe for study.

Perspectives

As the details of biogenesis and topogenesis of the components of each layer of the bacterial cell surface come into view, new questions of greater subtlety will arise: What feedback mechanisms coordinate the rates of synthesis and transport of components to each layer? How is compartmentation maintained during cell division? How are chromosome segregation and the locus of septation sited on the rod-shaped cell envelope? The central role of microbes in evolution and biodiversity and the desparate need for new targets for antibiotics are driving a renaissance in microbial research. The gram-negative envelope, as a uniquely bacterial structure, will continue to be at the center of these studies.

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